THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 29, Issue of July 19, pp. 26128–26135, 2002
Printed in U.S.A.

All Four Members of the Ten-m/Odz Family of Transmembrane Proteins Form Dimers*

Received for publication, April 17, 2002
Published, JBC Papers in Press, May 8, 2002, DOI 10.1074/jbc.M203722200

Kang Feng‡, Xiao-Hong Zhou‡§, Toshitaka Oohashi¶, Matthias Mörgelin‖, Ariel Lustig, Satoshi Hirakawa¶, Yoshifumi Ninomiya¶, Jürgen Engel**, Uwe Rauch‡, and Reinhard Fässler‡ ‡‡§§**

*From the ‡Department of Experimental Pathology and the ‖Department of Cell and Molecular Biology, Section for Connective Tissue Biology, Lund University, S-221 85 Lund, Sweden, the ¶Department of Molecular Biology and Biochemistry, Okayama University, Medical School, Okayama 700, Japan, the **Biozentrum, University of Basel, CH-4056, Basel, Switzerland, and the ‡‡Max Planck Institute for Biochemistry, D-82152 Martinsried, Germany*

Ten-m/Odz/teneurins are a new family of four distinct type II transmembrane molecules. Their extracellular domains are composed of an array of eight consecutive EGF modules followed by a large globular domain. Two of the eight modules contain only 5 instead of the typical 6 cysteine residues and have the capability to dimerize in a covalent, disulfide-linked fashion. The structural properties of the extracellular domains of all four mouse Ten-m proteins have been analyzed using secreted, recombinant molecules produced by mammalian HEK-293 cells. Electron microscopic analysis supported by analytical ultracentrifugation data revealed that the recombinant extracellular domains of all Ten-m proteins formed homodimers. SDS-PAGE analysis under nonreducing conditions as well as negative staining after partial denaturation of the molecules indicated that the globular COOH-terminal domains of Ten-m1 and -m4 contained subdomains with a pronounced stability against denaturing agents, especially when compared with the homologous domains of Ten-m2 and -m3. Cotransfection experiments of mammalian cells with two different extracellular domains revealed that Ten-m molecules have also the ability to form heterodimers, a property that, combined with alternative splicing events, allows the formation of a multitude of molecules with different characteristics from a limited set of genes.

The Ten-m/Odz protein was first found in *Drosophila* where it was proposed to be either a secreted tenascin-like molecule (1) or type I transmembrane receptor (2). We have subsequently identified and characterized the mouse Ten-m and found that it characterizes a new family of genes composed of 4 members (Ten-m1–4). The biochemical analysis of recombinant fragments of mouse Ten-m1 and alkaline phosphatase fusion proteins revealed that Ten-m1 is expressed as a type II transmembrane molecule. Furthermore, we could demonstrate that two of the eight tandemly arranged EGF[1] modules present in the extracellular domain of mouse Ten-m1 containing 5 instead of 6 cysteine residues facilitate the dimerization of two molecules in a covalent, disulfide-linked fashion. Members of the Ten-m family have in the meantime also been described in rat (3), chicken (4–7), human (8, 9), zebrafish (10), and *Caenorhabditis elegans* (11).

The expression pattern of Ten-m/Odz in flies and mammals suggests important roles during as well as after development. In *Drosophila* embryogenesis, Ten-m/Odz is expressed in seven stripes during the blastoderm stage (12) and later also in the central nervous system (1), heart (2), and eye (13). Expression studies of Ten-m1–4 in adult mouse tissues showed a widespread expression with the highest levels in the brain (14, 15). In chicken, both teneurin-1 (corresponding to Ten-m1) and teneurin-2 (corresponding to Ten-m2) are expressed in neurons of the developing visual system (4). Furthermore, teneurin-2 mRNA and protein are also found in the developing limbs, somites, and craniofacial mesenchyme (7). During the segmentation period of zebrafish, Ten-m3 is expressed in the somites, notochord, pharyngeal arches, and the brain, whereas the expression of Ten-m4 is restricted mainly to the brain (10).

Genetic studies of the fly Ten-m/Odz revealed a crucial role during segmentation and identified the Ten-m/Odz gene as the first pair rule gene that does not encode a transcription factor. Loss of Ten-m/Odz results in a typical deletion of cuticle segments, which appear in a reiterative manner along the body axis of the hatched larvae (1). The function of Ten-m/Odz genes in vertebrates, however, is unknown. It has been reported that various forms of stress including alkylating agents or UV light can trigger the activation of mouse Ten-m/Odz 4 (16). The induction of rat neurestin (corresponding to Ten-m2/Odz 2) in external tufted cells during regeneration of olfactory sensory neurons suggests a possible function in synapse formation and morphogenesis (3). Ectopic expression of a splice variant of teneurin-2 in neuronal cells significantly increased the number of filopodia and the formation of enlarged growth cones (5), suggesting a role in actin dynamics.

All four mouse Ten-m protein chains are 2700–2800 amino acids long and lack signal peptides at the NH_2 terminus, but they contain short hydrophobic stretches characteristic of transmembrane proteins. These hydrophobic domains are present about 300–400 amino acids after the translation start.

* The work was funded by the Swedish Science Foundation, the Göran Gustafsson Foundation for Research in Natural Science and Medicine, the Carl Tesdorpfs stiftelse, and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ A Bluecher fellow.

§§ To whom correspondence should be addressed: Max Planck Institute for Biochemistry, Am Klopferspitz 18a, D-82152 Martinsried, Germany. Tel.: 49-89-8578-2897; Fax: 49-89-8578-2422; E-mail: faessler@biochem.mpg.de.

[1] The abbreviations used are: EGF, epidermal growth factor-like; NEM, *N*-ethylmaleimide; GdmCl, guanidinium chloride; Ni-NTA, nickel-charged nitrilotriacetic acid; His, hexahistidine; mAb, monoclonal antibody; CMV, cytomegalovirus.

Approximately 200 amino acids COOH-terminal to this transmembrane region are eight consecutive EGF-like repeats. In all Ten-m/Odz genes the second as well as the fifth EGF module contain an odd number of cysteine residues. They mediate the covalent dimerization of two Ten-m proteins. The sequence similarity of the EGF repeats between the mouse Ten-m homologues ranges from 65 to 72%, whereas other parts are less conserved. The large COOH-terminal domains distal to the EGF repeats, for example, have similarities ranging between 58 and 68% (14). It has been shown recently for chicken teneurin-2 that the large COOH-terminal domain, constituting about 70% of the molecular mass, can be spliced alternatively (7). Outside of the EGF repeats, the Ten-m/Odz family sequences bear no similarity to any other eukaryotic sequences (15). However, the COOH-terminal part harbors 26 repetitive sequence motifs termed YD repeats, which are most similar to the core of the *rhs* elements of *Escherichia coli*. Related repeats in toxin A of *Clostridium difficile* bind specific carbohydrates (4).

In the present study, we characterized the properties of the extracellular domains of all four mouse Ten-m/Odz family members. They have essentially identical arrays of EGF repeats but show different cysteine patterns in the appending COOH-terminal domains. Ten-m2 and Ten-m4 contain an uneven number of this amino acid. We show that the recombinantly produced extracellular domains of Ten-m1–4 can form homodimers. Differences in the cysteine patterns in the globular COOH-terminal domains appear to affect the stability of the tertiary structures, whereas all four mouse Ten-m molecules share the same dimeric quaternary structure. In addition, we demonstrate that the Ten-m molecules have the ability to form heterodimers, a property allowing the formation of a multitude of molecules from a limited set of genes.

EXPERIMENTAL PROCEDURES

The Recombinant Expression of the Extracellular Domains of the Ten-m Proteins—The extracellular domains of Ten-m2 (starting at serine 572), Ten-m3 (starting at glutamic acid 513), Ten-m4 (starting at serine 564), and the globular COOH-terminal parts of Ten-m1 (Gten-m1; starting at glutamic acid 799) and Ten-m3 (Gten-m3; starting at glutamic acid 787) (Table I) were linked to the signal peptide of BM-40 via the sequence APLA (Ten-m3) (17) or APLGRGSHHHHHHGGLA (Ten-m2, Ten-m4, Gten-m1, and Gten-m3), which can be detected by the anti-RGS (H4) antibody (Qiagen). The latter property allows affinity purification on Ni-NTA-Sepharose (Qiagen) (18). The DNA was inserted into a eukaryotic expression plasmid driven by a CMV promoter (pRC/CMV, Invitrogen) and containing a puromycin resistance gene (19). Upon transfection into human embryonic kidney cells (HEK-293 cells, American Type Culture Collection) using LipofectAMINE (Invitrogen) puromycin-resistant clones were isolated as described earlier (14). Positive clones were identified by 5% SDS-PAGE and Coomassie Blue staining or Western blotting using mouse anti-RGS (H4) antibody (Qiagen, Stockholm, Sweden). For heterodimer analysis HEK-293 cells expressing the His-tagged extracellular domain of Ten-m2 were co-transfected with the non-His-tagged Ten-m1 or Ten-m3 extracellular domains, respectively, using the same eukaryotic expression vector described above (pRC/CMV, Invitrogen) but containing a neomycin resistance gene. Clones resistant to puromycin (1 μg/ml) as well as G418 (1.2 mg/ml) were identified by Western blot.

Monoclonal Antibodies against the Recombinant Ten-m1 and Ten-m3—The procedure has been described as the rat lymph node method (20) for raising monoclonal antibodies (mAb). Briefly, WKY/NCRj rats (Charles River Japan, Yokohama, Japan) were immunized in the hind footpads with the emulsion of the recombinant protein and Freund's complete adjuvant. Three weeks later the rats were killed, and lymphocytes from the medial iliac lymph nodes were fused with mouse myeloma cells (SP2/0-Ag14).

Supernatants from hybridoma cultures were screened by enzyme-linked immunosorbent assay using the recombinant protein as immobilized ligand. A subsequent screening was performed by indirect immunofluorescence for Ten-m1 and Ten-m3 using sections from mouse testis and mouse brain, respectively.

The specificity of the mAbs TO4 and HG31, which were raised against the extracellular domains of Ten-m1 and Ten-m3, respectively, were tested by Western assays using the recombinant extracellular domains of Ten-m1, Ten-m2, and Ten-m3. Briefly, 50 ng of purified recombinant protein was separated under nonreducing condition on 6% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences) in Tris/glycine buffer containing 10% methanol for 1 h with 100 V using the Bio-Rad mini-gel system. The membranes were blocked with 5% nonfat dry milk in TBST (20 mM Tris-HCl, pH7.6, 150 mM NaCl, 0.1% Tween 20), incubated with protein G-purified TO4 (1:1000) and HG 31 supernatant (1:3000), respectively, and developed with horseradish peroxidase-conjugated secondary antibody in TBST containing 5% nonfat dry milk and the ECL$^+$ detection system (Amersham Biosciences).

Purification of the His-tagged Secreted Proteins—Serum-free conditioned medium was dialyzed (three times, 6 h each time) against 50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 0.5 mM NEM, and 1 mM imidazole supplemented with freshly added 0.5 mM phenylmethylsulfonyl fluoride. 1 ml of the Ni-NTA slurry was added to 50 ml of conditioned medium, incubated with Ni-NTA matrix at 4 °C for at least 6 h, loaded into an empty column, and washed 15 times the column volume with wash buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 20 mM imidazole, 0.5 mM NEM). The protein was finally released with 5 ml elution buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 250 mM imidazole, 0.5 mM NEM). The eluate was concentrated to less than 1 ml by centrifugation through membranes with a cut-off of 10 kDa (Amicon). The concentrated protein was applied to a Superose 6 column equilibrated with 10 mM HEPES, pH 7.4, 500 mM NaCl, and 0.5 mM NEM. Fractions containing the purified molecules were dialyzed against with 10 mM HEPES, pH 7.4, 150 mM NaCl and stored at −80 °C.

Heterodimer Analyses of Recombinant Ten-m1 and Ten-m2 or Ten-m3 and Ten-m2 in Vitro—For heterodimer analysis of recombinant Ten-m1 and Ten-m2, 1 ml of conditioned medium from the co-transfected cells was dialyzed against 50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 0.5 mM NEM, and 1% bovine serum albumin plus 20 mM imidazole, each for 6 h for three times at 4 °C and then incubated with Ni-NTA beads overnight. The beads were washed with the same buffer containing 20 mM imidazole and finally eluted two times with the same buffer containing 250 mM imidazole. Specific bands were detected with TO4 (1:1000) mAb and anti-RGS (H4) (1:50,000) antibody on Western blot.

To analyze the heterodimers of the extracellular domains of Ten-m3 and Ten-m2, 1 ml of serum-free conditioned medium of cotransfected cells or a mixture of 0.5 ml of recombinant Ten-m3 and 0.5 ml of recombinant His-tagged Ten-m2 was applied to Ni-NTA beads. The beads were washed with 20 mM imidazole and finally eluted two times with 250 mM imidazole. Bands were detected with HG31 (1:3000) mAb and anti-RGS (H4) (1:50,000) antibody by Western blot.

Enzymatic Modification—*N*-glycosidase F (Roche Molecular Biochemicals) treatment (2 units) of 12 μg of purified recombinant extracellular domain of Ten-m1 or Ten-m2 was carried out at 37 °C for 16 h in 20 mM phosphate buffer, pH 7.2, containing 0.5% octylglucoside, 1 mM phenylmethylsulfonyl fluoride, and 10 mM EDTA. Subsequently the samples were denatured by heating to 97 °C for 5 min in the presence of 1% SDS in 20 mM phosphate buffer, pH 7.2.

Analytical Ultracentrifugation—A Beckman model XLA analytical Ultracentrifuge equipped with absorption optics was employed. Sedimentation velocity runs were performed in 12-mm double sector cells at rotor speeds of 40,000 and 52,000 rpm. Sedimentation equilibrium runs were performed at 4,400 rpm using the same cells but at a filling height of 1.5–3 mm only. Sedimentation coefficients $s_{20,w}$ are corrected to standard conditions (water at 20 °C) (21). The molecular masses, M, were calculated from sedimentation equilibrium runs using a floating base-line computer program that adjusts the base-line absorption to obtain the best linear fit of ln A *versus* r^2 (A = absorbance, r = distance from the rotor axis). A partial specific volume of 0.70 cm^3/g was used, which was calculated for proteins with 30% glycosylation (22). Frictional ratios f/f_0 were calculated from the sedimentation coefficients and molecular masses according to Van Holde (21), and axial ratios of ellipsoids of revolution a/b were determined from Perrin's table (21). All measurements were performed in 10 mM HEPES, 150 mM NaCl at 20 °C.

Electron Microscopy—Glycerol spraying/rotary shadowing, negative staining, and evaluation of the data from electron micrographs were carried out as described previously (23). For negative staining 5-μl samples of different Ten-m preparations (typical concentrations of about 10 μg/ml in Tris-buffered saline) were adsorbed to 400-mesh carbon-coated copper grids, washed briefly with water, and stained on two drops of freshly prepared 0.75% uranyl formate. The grids were

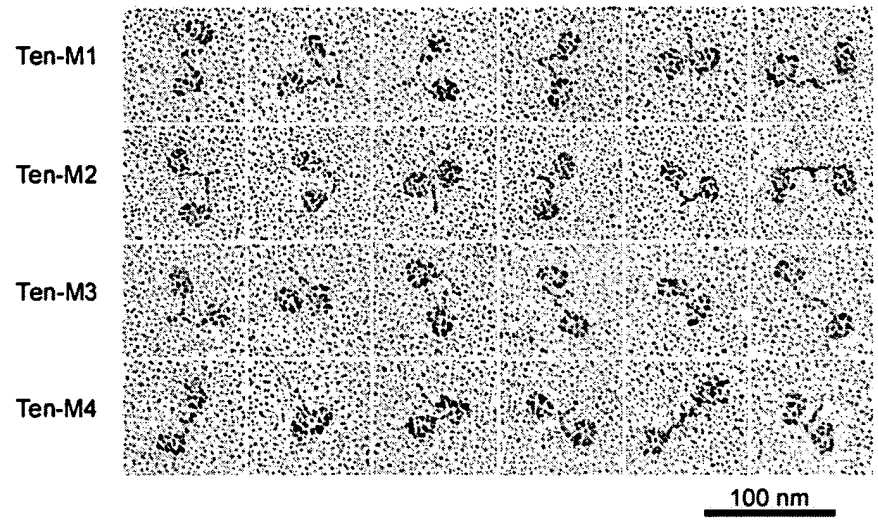

FIG. 1. **Rotary shadowing of purified recombinant extracellular domains of Ten-m1–4.**

rendered hydrophilic by glow discharge at low pressure in air. For glycerol spraying/rotary shadowing, Ten-m samples were dialyzed overnight at 4 °C against 0.2 M ammonium hydrogen carbonate, pH 7.9. They were mixed with equal volumes of 80% glycerol and sprayed onto freshly cleaved mica pieces with a nebulizer designed for small volumes. They were dried in a high vacuum for 2 h and shadowed under rotation with 2 nm platinum/carbon at a 9° angle, followed by coating with a stabilizing 10-nm carbon film. Specimens were observed in a Jeol JEM 1230 electron microscope operated at 80 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera. Molecular masses of globular protein domains from negatively stained images were estimated as described previously (23).

RESULTS

Properties of Purified Recombinant Mouse Ten-m Proteins—The extracellular domains of Ten-m2, -m3 and -m4, starting with the first EGF module, were recombinantly expressed in HEK-293 cells and purified from serum-free conditioned culture medium. The rotary shadowing electron microscopic image showed that the recombinant extracellular domain of all mouse Ten-m family members form similar cherry-like structures of two globular domains connected by two extended rods (Fig. 1) as previously observed for the recombinant extracellular domain of Ten-m1 (14). In some images the connecting part between the two globular domains was extremely extended. In such cases the distances between the two globular domains was up to 30 nm (Fig. 1).

Separation of all four recombinantly expressed extracellular domains of the Ten-m family proteins by 6% SDS-PAGE under reducing conditions revealed apparent molecular masses of about 225 kDa for all four Ten-m proteins (Fig. 2*A*). Gel separation under nonreducing conditions showed significant differences in the migratory behavior of the four samples (Fig. 2*B*) dividing the extracellular domains of the four Ten-m molecules into two subfamilies, one consisting of Ten-m1 and Ten-m4 (Fig. 2*B*, *lanes 1* and *2*), which migrate considerably slower on a 6% SDS-PAGE than the second subfamily, consisting of Ten-m2 and Ten-m3 (Fig. 2*B*, *lanes 3* and *4*).

In our previous study (14) Ten-m1 was proposed to be a dimer mainly on the basis of electron microscopic observations, although a thorough SDS-PAGE analysis with technically adequate markers had not been performed. Application of a 3–12% gradient SDS-PAGE allowed the extracellular domains of Ten-m1 to migrate 4.5 cm into the polyacrylamide gel and decreased the apparent differences between the migratory abilities of the extracellular domains of the Ten-m molecules (Fig. 2*C*). Apparent molecular masses derived from this gel using laminin (850 kDa), nidogen (150 kDa), and myosin (200 kDa) for calibration were 550 kDa for Ten-m1 and Ten-m4 and 440 kDa for Ten-m2 and Ten-m3. For the later proteins an additional band was seen migrating with the same apparent molecular mass of 225 kDa as the reduced protein, thus probably corresponding to unlinked monomers.

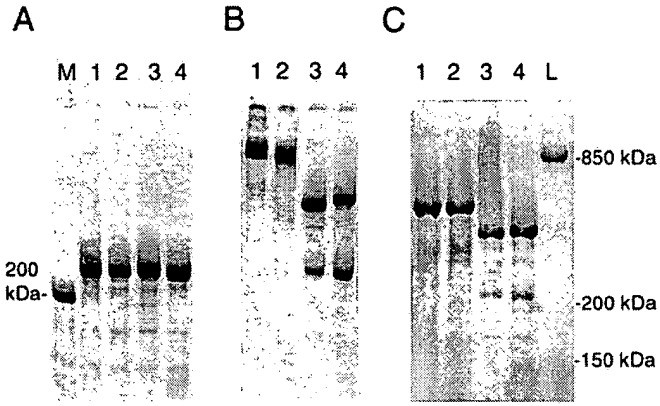

FIG. 2. **Gel separation of the recombinant extracellular domains of Ten-m1–4 by SDS-PAGE.** The extracellular domains of Ten-m1 (*lanes 1*), Ten-m4 (*lanes 2*), Ten-m2 (*lanes 3*), and Ten-m3 (*lanes 4*) were separated on a 6% SDS-PAGE (*A* and *B*) or on a 3–12% gradient SDS-PAGE (*C*) under reducing (*A*) or nonreducing (*B* and *C*) conditions. Additional *lanes* show myosin (*M*) and laminin/nidogen (*L*), which were used as molecular mass markers.

To test whether differential *N*-linked glycosylation was responsible for the observed differences, the recombinant extracellular domains of Ten-m1 and Ten-m2 were subjected to *N*-glycosidase F treatment. This treatment reduced the apparent molecular masses of both molecules to a similar extent on SDS-PAGE ruling out *N*-linked glycosylation as the cause of the different migratory behaviors (results not shown). Despite their different migratory abilities on SDS-PAGE, the extracellular domains of Ten-m proteins from the two different subfamilies had identical elution profiles when subjected to gel permeation chromatography on Superose 6 (results not shown).

It is possible that molecular mass determination on SDS-PAGE under nonreducing conditions might be affected by particular tertiary or quaternary structures, which could be expected to be more different in molecules with unrelated sequences, like Ten-m 1 and laminin, than in molecules with closely related sequences, like Ten-m1 and Ten-m2. To obtain an accurate molecular mass independent of the structural peculiarities of the subjected substance, we performed ultracentrifugation experiments. The equilibrium ultracentrifugation data showed that both the recombinant extracellular domains

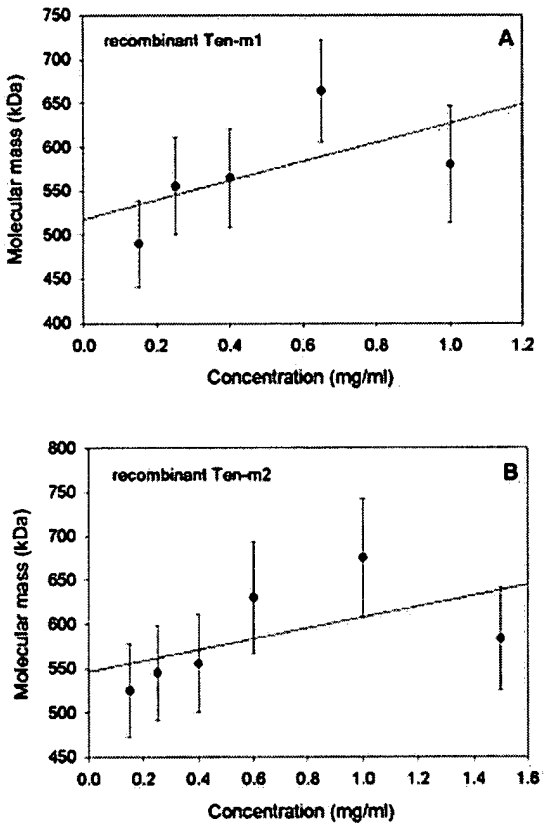

FIG. 3. **Molecular masses determination of the extracellular domains of Ten-m1 (*A*) and Ten-m2 (*B*) under nonreducing conditions by equilibrium centrifugation.** Experimental values at different protein concentrations are extrapolated to zero concentration by linear regression. Standard deviations of ±10% are indicated by *error bars*.

of Ten-m1 and Ten-m2 had under nonreducing condition approximately the same average molar mass of 500–550 kDa. Experimental values are M = 515,000 ± 60,000 for Ten-m1 and 545,000 ± 60,000 for Ten-m2 (Fig. 3). The comparison of these values with the calculated molecular mass of a single polypeptide chain of the recombinantly expressed sequence of Ten-m1 (247 kDa) and Ten-m2 (245 kDa), both containing 13 potential *N*-glycosylation sites (Table I), indicates that the extracellular domains of both Ten-m proteins assume a similar dimeric quaternary structure. This result underlines the observations using rotary shadowing electron microscopy (Fig. 1) and indicates that, despite their differences in mobility on SDS-PAGE, all Ten-m molecules are dimeric type II transmembrane molecules covalently linked only via their EGF module arrays. Sedimentation velocity experiments with the recombinant extracellular domains of Ten-m1 and Ten-m2 gave sedimentation coefficient values of $s_{20,w}$ = 15.4 and 16.2 S, respectively. Frictional ratios calculated with these values and M = 500,000 are f/f_0 = 1.23 and 1.17, respectively. This indicates an axial ratio a/b of about 4 for the hydrodynamic equivalent of the dimers, which is consistent with the asymmetric shape revealed by electron microscopy (see below).

Because the EGF module arrays are best conserved in all Ten-m molecules (14), they are least likely to account for biophysical differences between the subfamilies. To test whether the large COOH-terminal globular domain following the EGF module array was responsible for the differences in migratory behavior, the COOH-terminal domain of one member of each subfamily of Ten-m1 and Ten-m3 was recombinantly expressed and analyzed. As observed previously, the secreted protein products showed a different migratory behavior on SDS-PAGE. Although the migration behavior of the COOH-terminal domain of Ten-m3 (Gten-m3) was similar under reducing and nonreducing condition, the COOH-terminal region of Ten-m1 (Gten-m1) migrated considerably slower under nonreducing conditions on a 6% SDS-PAGE (Fig. 4, *A* and *B*). Again, on a 3–12% gradient gel the difference in migratory behavior between the nonreduced molecules appeared decreased (Fig. 4*C*). The apparent molecular masses determined from this gel were 195 kDa for Gten-m3 and 300 kDa for Gten-m1, with a second, less distinct subpopulation of Gten-m1 of about 250 kDa, most likely representing proteolytically nicked material. Thus, the observed reduced mobility of the extracellular domain of Ten-m1 on SDS-PAGE was reflected in a similarly reduced mobility of its globular COOH-terminal part.

The electron microscopic analysis of Ten-m1 by negative staining revealed that the large, globular COOH-terminal domain is composed of subdomains (14). The subdomains were especially evident in preparations of the globular COOH-terminal domains alone (Fig. 4*D*). In Gten-m1 preparations we could almost always observe two globular structures, whereas in Gten-m3 preparations we observed mainly single globular structures. After incubation with 4 M GdmCl, a condition known to disrupt most noncovalent protein interactions, we observed only single globular domains in Gten-m3 samples, whereas structures with two globules could still be observed in Gten-m1 preparations. A morphometric analysis of those two globular subdomains in the Gten-m1 preparation indicated molecular mass values of 40 to 60 kDa.

Biochemical Analysis of Ten-m Heterodimers in Vitro—The expression patterns of the four Ten-m genes partially overlap in embryonic and adult tissues.[2] Because the EGF domains, which are responsible for the dimerization, are the best conserved part of all family members (14), we hypothesized that Ten-m proteins may also form heterodimers. To test whether the extracellular domains of the Ten-m proteins have the ability to dimerize with each other, we co-transfected and expressed the extracellular domain of Ten-m1 and a His-tagged extracellular domain of Ten-m2 in HEK-293 cells. Subsequently the supernatant was applied to a Ni-NTA column to purify His-tagged molecules. To control the assay conditions, supernatants derived from HEK-293 cells expressing either only the extracellular domain of Ten-m1 or only the His-tagged extracellular domain of Ten-m2, respectively, were mixed to obtain a similar ratio of Ten-m1 and Ten-m2 as expressed by the co-transfected cells. The mixed supernatants were subjected to the same purification procedure. Because on a 6% SDS-PAGE the differences in the migration behavior between homodimers of the extracellular domain of Ten-m1 and Ten-m2 were most evident (Fig. 2*B*), the eluates from the Ni-NTA were analyzed on a 6% SDS-PAGE (Fig. 5). The eluate from a mixture of supernatants with either recombinant Ten-m1 or His-tagged Ten-m2 extracellular domains contained only one major protein band, corresponding to the size of a Ten-m2 homodimer. In the eluates from the supernatants of the co-transfected cells, an additional band was visible that migrated between the Ten-m1 and Ten-m2 homodimers (Fig. 5). This observation suggested that heterodimerization of Ten-m1 and Ten-m2 can occur.

To confirm the observation, we generated mAbs against the recombinant extracellular domains of Ten-m1 and Ten-m3 to be able to identify protein bands by Western blot. The mAb TO4 was raised against Ten-m1 and showed very weak cross-reactivity with Ten-m2 and Ten-m3, whereas mAb HG31 raised

[2] X.-H. Zhou, K. Feng, T. Oohashi, K. Campbell, Y. Ninomiya, U. Rauch, and R. Fässler, manuscript submitted for publication.

TABLE I

Predicted characteristics of the proteins expressed in HEK-293 cells

The sequences derived from the respective mouse Ten-m cDNAs starts with the NH_2-terminal amino acid as indicated. The NH_2-terminal sequence is derived from the proteolytic processing site of the BM 40 signal peptide (17). The molecular mass was calculated on the basis of the NH_2-terminal sequence and the Ten-M C-termini predicted from the respective cDNA sequences. The number of potential *N*-glycosylation sites is based on the N-*X*-S/T recognition motif excluding proline residues at position 2.

Construct	NH_2-terminal amino acid	NH_2-terminal sequence	Calculated molecular mass	Potential *N*-glycosylation sites
Ten-m1	E 526	APLAE IMDDC STNCN GNGEC	247	13
Ten-m2	S 572	APLGR GSHHH HHHGG LASVQ	245	13
Ten-m3	E 513	APLAE SVVEC PRNCH GNGEC	246	13
Ten-m4	S 564	APLGR GSHHH HHHGG LASVD	248	11
Gten-m1	E 799	APLGR GSHHH HHHGG LAEML	220	13
Gten-m3	E 787	APLGR GSHHH HHHGG LAETL	219	13

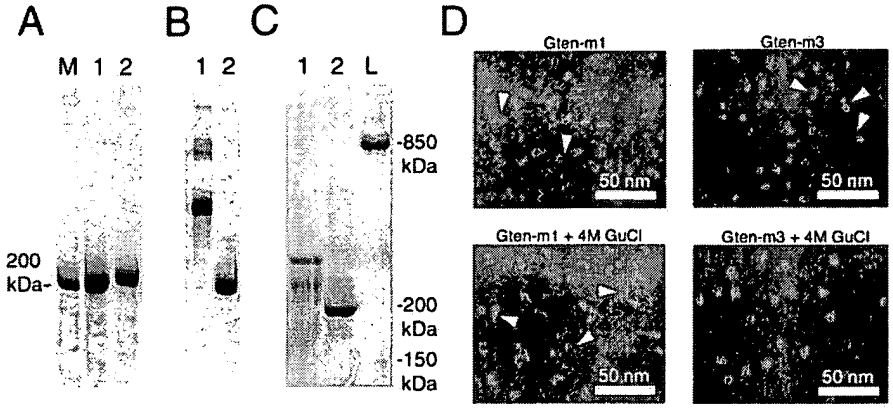

FIG. 4. **Gel separation and negative staining of the recombinant globular domains of Ten-m 1 and Ten-m3.** *A–C*, Coomassie Blue staining of the globular domains of Ten-m1 (*Gten-m1, lanes 1*) and Ten-m3 (*Gten-m3, lanes 2*) separated on a 6% SDS-PAGE (*A* and *B*) or on a 3–12% gradient SDS-PAGE (*C*) under reducing (*A*) or nonreducing (*B* and *C*) conditions. Additional *lanes* show myosin (*M*) and laminin/nidogen (*L*), which were used as molecular mass markers. *D*, negative staining of G-ten-m1 and G-ten-m3 with and without 4 M GdmCl. *White arrowheads* point to two subdomains of G-ten-m1 (*upper left panel*), two subdomains of G-ten-m3 (*upper right panel*), and two subdomains of G-ten-m1 with 4 M GdmCl (*lower left panel*).

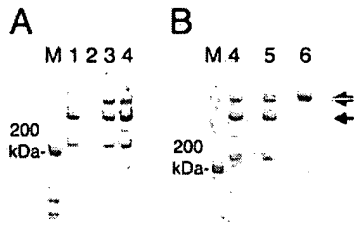

FIG. 5. **Heterodimerization of the extracellular domains of Ten-m1 and Ten-m2.** *A*, eluates of Ni-NTA beads loaded with a mixture of supernatants derived from cells transfected with an expression plasmid encoding the non-His-tagged extracellular domain of Ten-m1 and His-tagged extracellular domain of Ten-m2, respectively (*lane 1*); supernatant derived from cells transfected exclusively with an expression plasmid encoding the non-His-tagged extracellular domain of Ten-m1 (*lane 2*); or supernatants derived from two distinct cell clones co-transfected with expression plasmids encoding the non-His-tagged extracellular domain of Ten-m1 as well as the His-tagged extracellular domain of Ten-m2 (*lanes 3* and *4*). *B*, *lane 4* shows the same material as *lane 4* in *A*, with the heterodimer of the extracellular domains of Ten-m1 and Ten-m2 indicated by the *lower half arrow* and the homodimer of the extracellular domain of Ten-m2 indicated by a *full arrow*. *Lane 6* shows purified recombinant extracellular domain of Ten-m1 alone indicated by the *upper half arrow*. *Lane 5* is a mixture of the samples loaded in *lanes 4* and *6* showing the strongly stained heterodimer (*lower half arrow*) and purified recombinant extracellular domain of Ten-m1, which is weakly visible above (*upper half arrow*). *Lanes* labeled with *M* show myosin as the molecular mass (200 kDa) marker.

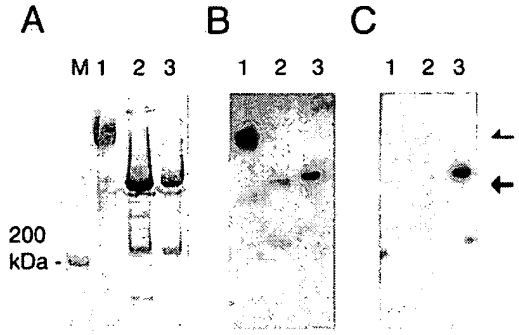

FIG. 6. **Specificity of monoclonal antibody TO4 and HG31.** *A*, *lanes 1–3* show 2 μg of purified recombinant extracellular domains of Ten-m1 (*upper half arrow*), His-tagged Ten-m2 (*full arrow*), and Ten-m3, respectively, separated under nonreducing condition on 6% SDS-PAGE and stained with Coomassie Blue. The *lane* labeled *M* shows myosin used as the molecular mass marker. *B* and *C*, two sets of 50 ng of the same three molecules were separated in parallel under the same conditions as in *A*, transferred to a polyvinylidene difluoride membrane and detected with the monoclonal antibodies TO4 and HG31, respectively.

against Ten-m3 showed no cross-reactivity with Ten-m1 and Ten-m2 (Fig. 6). The epitopes for both antibodies were localized within the eight EGF modules and were sensitive to reducing agents (results not shown). A third, commercial antibody used in our investigations recognizes the RGSHHHH sequence present in the His tag of Ten-m2. Using these antibodies we subjected the supernatant derived from co-transfected cells to a Ni-NTA affinity chromatography and were able show that the retained proteins migrating between Ten-m1 and Ten-m2 homodimers were recognized by the TO4 (anti-Ten-m1) as well as by the anti-RGS (H4) antibody (detecting His-tagged Ten-m2; Fig. 7, *lanes 4*). These findings further supported the notion that mammalian cells are able to express Ten-m1/Ten-m2 heterodimers.

Next we tested whether Ten-m members belonging to the

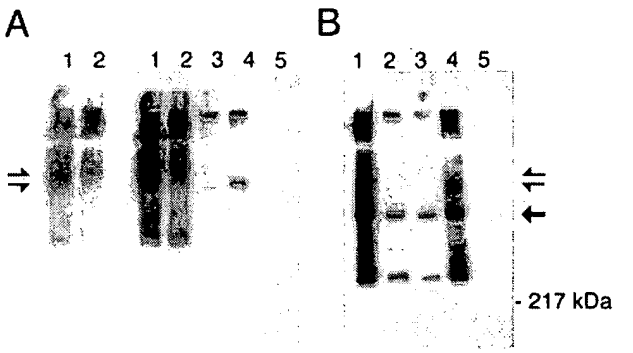

FIG. 7. **Ten-m1 and Ten-m2 heterodimers.** *A*, Western blot with the monoclonal antibody TO4 of supernatant of 293 cells co-transfected with the expression plasmid encoding the extracellular domain of Ten-m1 and the His-tagged extracellular domain of Ten-m2 before (*lanes 1*) and after (*lanes 2*) incubation with Ni-NTA beads. *Lanes 1* and *2* to the *left* have a shorter exposure time showing more clearly a loss of the faster migrating components (*lower half arrow*) after incubation with the beads. *Lane 3* shows material washed out from the Ni-NTA beads with 20 mM imidazole, and *lanes 4* and *5* show the first and second eluates from Ni-NTA beads with 250 mM imidazole. *B*, Western blot of the same samples shown in *A* developed with anti-RGS (H4) antibody. The *full arrow* indicates the position of the His-tagged Ten-m2 homodimers.

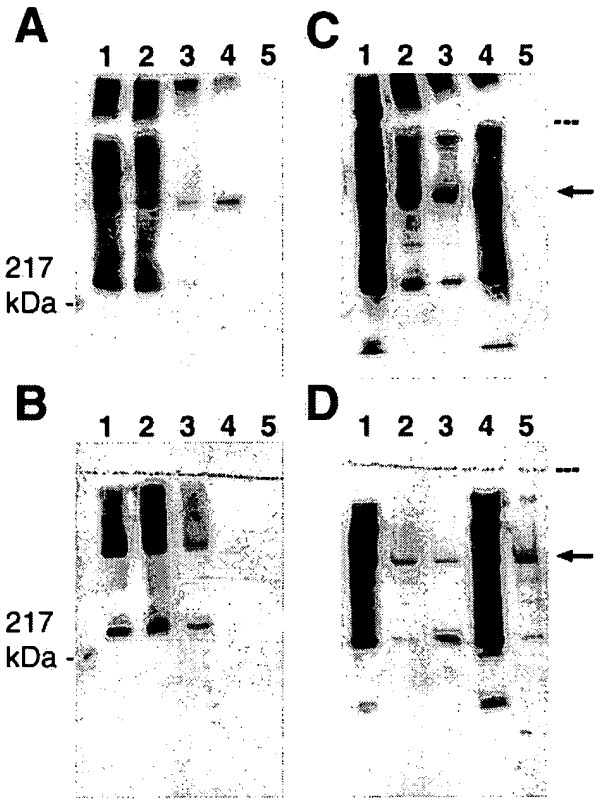

FIG. 8. **Ten-m2 and Ten-m3 heterodimers.** *Panels A* and *C* show samples derived from cells co-transfected with expression plasmids encoding the His-tagged Ten-m2 and non-His-tagged Ten-m3 extracellular domains. *Panels B* and *D* show mixed supernatants derived from cells singly transfected with expression plasmids encoding the His-tagged Ten-m2 and non-His-tagged Ten-m3, respectively. *Panels A* and *B* are developed with the monoclonal antibody HG31, and *panels C* and *D* are developed with the anti-RGS (H4) antibody. *Lane 1* represents the untreated supernatant, and *lane 2* shows the sample treated with the Ni-NTA beads (unbound material). *Lane 3* shows material washed out from the Ni-NTA beads with 20 mM imidazole, and *lanes 4* and *5* show the first and second eluates from Ni-NTA beads washed with 250 mM imidazole. Note that the *band* in *lane 4* of *panel A* is absent in *lane 4* of *panel B*. The *full arrows* indicate the positions of the homo- or heterodimers.

same subgroup are also able to form heterodimers. We decided to co-transfect His-tagged Ten-m2 and non-His-tagged Ten-m3 extracellular domains and to analyze heterodimers by Ni-NTA chromatography and subsequent Western blots using the anti-RGS (H4) antibody detecting His-tagged Ten-m2 and mAb HG31 specific for Ten-m3. As done previously, we compared the Ni-NTA binding properties of the supernatant of co-transfected cells with the properties of a mixture of supernatants of either His-tagged Ten-m2 or Ten-m3 single transfected cells. Fig. 8 shows that only the imidazole eluate of the supernatant derived from co-transfected cells contains HG31-positive material but not the eluate of the mixed supernatants.

These data suggest that the extracellular domain of Ten-m2 is able to heterodimerize with both Ten-m1 and Ten-m3.

DISCUSSION

The characterization of recombinant extracellular domains of the Ten-m family proteins revealed that they share the same quaternary structure, a dimer composed of two large COOH-terminal domains and arrays of eight EGF modules cross-linked by two disulfide bridges (Fig. 9). The dimers are linked to the cell membrane by stretches of 170–200 amino acids without any cysteine residue (linker domain) and extended in the cytosol by intracellular NH_2-terminal polypeptides of 310–380 amino acids. In rotary shadowing experiments the COOH-terminal domains, which constitute about 70% of the mass of the molecule, appeared as one globular unit. Using negative staining it can be subdivided into at least two subdomains.

The cysteine and *N*-glycosylation pattern of the COOH-terminal globular domains of the mouse Ten-m molecules allows to divide them tentatively into three parts, a cysteine-rich part, an *N*-glycosylation-rich part, and a COOH-terminal part (Fig. 9). The COOH-terminal part is characterized by the presence of 4 conserved cysteines in Ten-m1 and Ten-m4, which are absent in Ten-m2 and Ten-m3. These conserved cysteine residues classify the four mouse Ten-m molecules in two subfamilies, which are identical to the two subfamilies observed by SDS-PAGE under nonreducing conditions. Using SDS-PAGE, we found that the extracellular domains of Ten-m1 and Ten-m4 migrate significantly slower than the domains of Ten-m2 and Ten-m3. This observation indicates that the cysteines conserved in Ten-m1/4 and missing in Ten-m2/3 might increase the stability of a protein fold to a level at which this structure resists even the normally highly denaturing condition of 2% SDS. This hypothesis is supported further by the observation that the two globular structures in negatively stained samples of the COOH-terminal domain of Ten-m1 resist the treatment of 4 M GdmCl, but the same treatment destroys the folding of a second globular structure in the COOH-terminal domain of Ten-m3.

Interestingly, the connecting *N*-glycan-rich part of about 800 amino acids is essentially identical to the region of 26 simple repetitive YD motifs described by Minet *et al.* (4) in chicken teneurin-1, whereas the following 70-amino-acid long condensed YD repeats coincide with the beginning of the COOH-terminal part. YD repeat-containing bacterial proteins bind carbohydrates. The simple YD repeat part of teneurin-1 expressed in human fibrosarcoma cells can bind heparin and support the outgrowth of neurites from dorsal root ganglia in a heparin-sensitive fashion (4). In contrast to these findings, we found that the complete extracellular domain of mouse Ten-m1 has no significant affinity to a heparin matrix.[3]

The apparent molecular masses of all four extracellular do-

[3] K. Feng, X.-H. Zhou, T. Oohashi, M. Mörgelin, A. Lustig, S. Hirakawa, Y. Ninomiya, J. Engel, U. Rauch, and R. Fässler, unpublished observations.

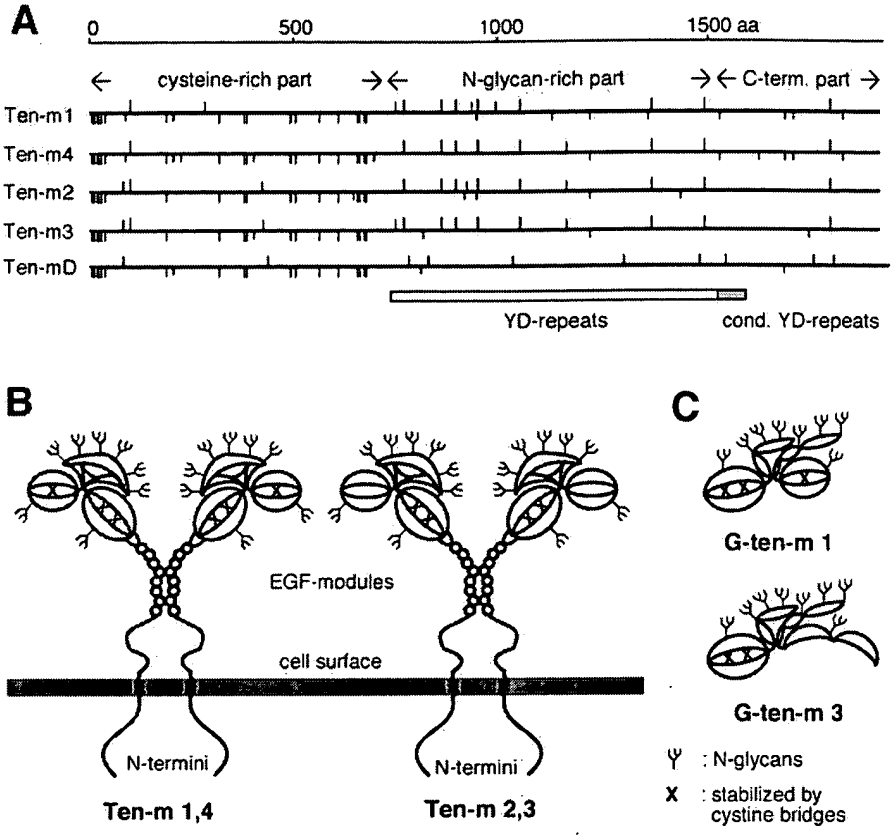

FIG. 9. **Proposed structural properties of the mouse Ten-m proteins.** *A*, line presentation of the COOH-terminal globular domains of the four mouse and the *Drosophila* (*Ten-mD*) Ten-m proteins showing the cysteines (*strokes below* the *line*, with *longer strokes* indicating conservation in all five proteins) and *N*-glycosylation sites (*strokes above* the *line*, with *longer strokes* indicating conservation in all four mouse proteins). The number of amino acids and the three proposed subdivisions within the COOH-terminal domain are indicated *above* and the location of YD repeats and condensed YD repeats *below* the *lines* representing the Ten-m globular domains. *B*, schematic presentation of the two subfamilies of mouse Ten-m molecules. Two polypeptide chains are inserted into the plasma membrane as type II transmembrane molecules and connected by the second and fifth EGF repeats. The COOH-terminal globular domains of Ten-m1 and Ten-m4 are represented by two strongly covalently stabilized (*X*) subdomains, whereas Ten-m2 and Ten-m3 have only one subdomain stabilized by intramolecular covalent bonds. *C*, subdomains not stabilized by intramolecular covalent bonds are more likely to lose their native structure. Thus, they would no longer be observed by negative staining under denaturing conditions and they would not interfere with the binding of SDS to the protein, which is a prerequisite for normal electrophoretic behavior.

mains observed by SDS-PAGE were lower than the values calculated from the recombinantly expressed amino acid sequences. This was also observed for the extracellular domain of Ten-m2 and Ten-m3 under nonreducing conditions and might be related to peculiarities in the amino acid composition of all four molecules affecting the association with dodecyl sulfate anions. In turn, the apparent molecular masses determined by equilibrium ultracentrifugation came closer to the presumably real values, composed of the calculated molecular masses and the contribution of the *N*-linked oligosaccharides (Table I); this demonstrates the superiority of this method, which is independent of structural or compositional peculiarities of the analyzed proteins.

The a/b of 4 determined by sedimentation velocity experiments is consistent with the shape of most molecules observed by rotary shadowing electron microscopy, showing two well separated globular structures linked by branching, interconnected rods. The observed distance of the globules was up to 30 nm. Thus, they were 15 nm away from the fifth EGF module, which is considered the branching point. The expected length for three tandemly arranged EGF modules is 6 nm. This may indicate that the first part of the COOH-terminal domain is composed of one or more small, independently folding structural unit(s) covering the remaining 9 nm distance. It has already been proposed previously that this small module, which contains 6 cysteines (therefore called the C-C domain) and immediately follows the EGF arrays, is an independently folding unit (1, 3, 16). However, convincing biochemical evidence for the existence of such a module is lacking.

We could demonstrate that heterodimerization of Ten-m molecules is possible in HEK-293 cells. Overlapping *in situ* hybridization and protein expression patterns indicate that more than one Ten-m molecule is expressed by the same cell *in vivo*. Similar properties have been reported for other protein families such as the matrilins (a family of extracellular matrix molecules), which can form heteromers by generating a tetramer consisting of two matrilin 1 and two matrilin 3 molecules. Interestingly, co-transfection experiments revealed that COS-7 cells also secreted those tetramers found *in vivo* but no heteromers that do not occur *in vivo* (24). This may indicate that the pattern of heteromeric molecules produced recombinantly in mammalian cells is likely to reflect their presence *in vivo*. However, we cannot rule out at the moment that mechanisms might have evolved *in vivo* that prevent the formation of Ten-m heteromers, for example by interactions of the cytoplasmic domains with molecules able to separate Ten-m polypeptides to different membrane compartments within the endoplasmic reticulum. So far no molecules have been reported that are able to interact with the cytoplasmic domains of the Ten-m molecules (4).

Our model for the Ten-m molecules proposes that the cytoplasmic domains are linked constitutively to each other by the extracellular part, unless one of the polypeptide chains connecting the EGF part with the cell membrane is cleaved proteolytically. A tribasic potential furin cleavage site has been identified and confirmed experimentally in teneurin-2, the chicken homologue of mouse Ten-m2. This site is also conserved in mouse Ten-m2 and rat neurestin. Therefore, it is possible that homodimeric Ten-m molecules may be cleaved, giving rise to a diffusible extracellular domain and two independent cytoplasmic membrane-linked fragments. Moreover, in heterodimers only one polypeptide chain might be susceptible to proteolytic cleavage, thereby separating just one cytosolic domain.

In summary, Ten-m proteins represent a family of dimeric molecules, which can be divided into at least five functional units: a cytoplasmic part, a transmembrane part, a linker region, a dimerization (EGF) unit, and a large globular COOH-terminal domain. The best conserved part among all homo- and orthologues is the dimerization unit, which is able to support the formation not only of homodimers but also of heterodimers.

This property allows the formation of a multitude of molecules from a limited set of genes.

Acknowledgment—We thank Dr. Rupert Timpl for careful reading of the manuscript.

REFERENCES

1. Baumgartner, S., Martin, D., Hagios, C., and Chiquet-Ehrismann, R. (1994) *EMBO J.* **13,** 3728–3740
2. Levine, A., Bashan-Ahrend, A., Budai-Hadrian, O., Gartenberg, D., Menasherow, S., and Wides, R. (1994) *Cell* **77,** 587–598
3. Otaki, J. M., and Firestein, S. (1999) *Dev. Biol.* **212,** 165–181
4. Minet, A. D., Rubin, B. P., Tucker, R. P., Baumgartner, S., and Chiquet-Ehrismann, R. (1999) *J. Cell Sci.* **112,** 2019–2032
5. Rubin, B. P., Tucker, R. P., Martin, D., and Chiquet-Ehrismann, R. (1999) *Dev. Biol.* **216,** 195–209
6. Tucker, R. P., Martin, D., Kos, R., and Chiquet-Ehrismann, R. (2000) *Mech. Dev.* **98,** 187–191
7. Tucker, R. P., Chiquet-Ehrismann, R., Chevron, M. P., Martin, D., Hall, R. J., and Rubin, B. P. (2001) *Dev. Dyn.* **220,** 27–39
8. Brandau, O., Schuster, V., Weiss, M., Hellebrand, H., Fink, F. M., Kreczy, A., Friedrich, W., Strahm, B., Niemeyer, C., Belohradsky, B. H., and Meindl, A. (1999) *Hum. Mol. Genet.* **8,** 2407–2413
9. Minet, A. D., and Chiquet-Ehrismann, R. (2000) *Gene* **257,** 87–97
10. Mieda, M., Kikuchi, Y., Hirate, Y., Aoki, M., and Okamoto, H. (1999) *Mech. Dev.* **87,** 223–227
11. Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., Coulson, A., Craxton, M., Dear, S., Du, Z., Durbin, R., *et al.* (1994) *Nature* **368,** 32–38
12. Baumgartner, S., and Chiquet-Ehrismann, R. (1993) *Mech. Dev.* **40,** 165–176
13. Levine, A., Weiss, C., and Wides, R. (1997) *Dev. Dyn.* **209,** 1–14
14. Oohashi, T., Zhou, X. H., Feng, K., Richter, B., Morgelin, M., Perez, M. T., Su, W. D., Chiquet-Ehrismann, R., Rauch, U., and Fässler, R. (1999) *J. Cell Biol.* **145,** 563–577
15. Ben-Zur, T., Feige, E., Motro, B., and Wides, R. (2000) *Dev. Biol.* **217,** 107–120
16. Wang, X. Z., Kuroda, M., Sok, J., Batchvarova, N., Kimmel, R., Chung, P., Zinszner, H., and Ron, D. (1998) *EMBO J.* **17,** 3619–3630
17. Mayer, U., Nischt, R., Pöschl, E., Mann, K., Fukuda, K., Gerl, M., Yamada, Y., and Timpl, R. (1993) *EMBO J.* **12,** 1879–1885
18. Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R. A., Nordheim, A., and Stunnenberg, H. G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 8972–8976
19. Kohfeldt, E., Göhring, W., Mayer, U., Zweckstetter, M., Holak, T. A., Chu, M. L., and Timpl, R. (1996) *Eur. J. Biochem.* **238,** 333–340
20. Kishiro, Y., Kagawa, M., Naito, I., and Sado, Y. (1995) *Cell Struct. Funct.* **20,** 151–156
21. Van Holde, K. E. (1971) *Physical Biochemistry*, pp. 98–120, Prentice-Hall, Englewood Cliffs, NJ
22. Ralson, G. (1993) *Introduction to Analytical Ultracentrifugation*, Beckman Instruments, Fullerton, CA
23. Engel, J., and Furthmayr, H. (1987) *Methods Enzymol.* **145,** 3–78
24. Zhang, Y., and Chen, Q. (2000) *J. Biol. Chem.* **275,** 32628–32634